NOTES

PHENOMYCIN, TOXICITY AND DISTRIBUTION

Shoshiro Nakamura
Pharmaceutical Institute, Hiroshima University School of Medicine, Kasumi-cho, Hiroshima, Japan

Tomio Takeuchi, Senji Hori, Meiki Matsuzaki and Hamao Umezawa
Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

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Phenomycin is a basic polypeptide antitumor antibiotic obtained from the cultured broth of *Streptomyces fervens* var. *phenomyceticus* and shows significant inhibitory activity against Ehrlich carcinoma, sarcoma 180 and adenocarcinoma 755 in mice[1,2,3]. The molecular weight of phenomycin is estimated to be 10,000 by Archibald's method and 10,000~10,500 by the amino acid analysis[4]. Molar ratio of the amino acids in phenomycin is as follows: aspartic acid 10~11, threonine 7, serine 7~8, glutamic acid 3~4, proline 4, glycine 4~5, alanine 19, valine 3, methionine 2, isoleucine 4, leucine 4, tyrosine 3, phenylalanine 1, tryptophan 1, lysine 14, histidine 4, arginine 5.

Phenomycin contains 4 moles of histidine and can be carboxymethylated with monoiodoacetic acid. The radioactive carboxymethylated phenomycin, prepared by reaction with $^{14}CH_2$-iodoacetic acid was employed for studies of its distribution in various organs of mice.

Aqueous solution of phenomycin and monoiodoacetic acid at various pHs (pH 6.0, 7.0, 8.0 and 9.0 respectively) were kept at 25°C for 24 hours. The resulting solutions were examined by electrophoresis on a cellulose acetate film using 0.1 M Tris-HCl buffer (pH 9.0) for 15 minutes at 8 mA/8 cm. It was shown that the reaction at various pH gave the same reaction products. Five purple-red spots, which moved toward the cathode 3.5, 3.2, 2.9. 2.6 and 2.3 cm, were detected from all of the reaction mixtures by treatment with Ponceu 3 R reagent, and the fastest spot was identical to that of phenomycin. The second spot was presumed to correspond to monocarboxymethylphenomycin (MCMP) and the other three to *di*-, *tri*- and *tetra*-carboxymethylphenomycin in decreasing order of movement. An aqueous solution of phenomycin and monoiodoacetic acid (pH 7.0) was kept at 25°C for 68 hours and the reaction products were separated on a column of CM-cellulose eluted with a gradient concentration of aqueous ammonium formate. The purity of each fraction was tested by electrophoresis. A mixture of *tetra*- and *tri*-substituted derivatives was eluted first, then the *di*-substituted derivative followed by monocarboxymethylphenomycin and finally phenomycin was eluted from the column. The gradient colum chromatography on CM-cellulose was repeated on the eluates until a single spot was observed by electrophoresis. Thus, pure MCMP and dicarboxymethylphenomycin were recovered from the reaction mixture.

MCMP and dicarboxymethylphenomycin were hydrolyzed with constant boiling hydrochloric acid in sealed tubes at 110°C for 17 hours. One mole of 3-monocarboxymethylhistidine and 3 moles of histidine were found in the hydrolyzate of MCMP by the Stein and Moore method. Two moles of histidine out of 4 moles of histidine in phenomycin were lost in the hydrolyzate of dicarboxymethylphenomycin.

Phenomycin prolonged the survival period of mice inoculated with 2 million cells of Ehrlich carcinoma and treated by daily intraperitoneal injection of 0.3 mcg/mouse for 1 week, while MCMP did not show antitumor activity by daily injection of 25 mcg/mouse for 1 week.

$^{14}CH_2$-MCMP (2.45×10^6 dpm/mg) was subcutaneously injected at 50 mg/kg into 4 mice (body weight, 20 g) to examine the distribution of MCMP in various tissues and organs[5]. Two mice were sacrified 1 hour after the injection and another two mice 6 hours after the injection. Each tissue or organ was separated and centrifuged after grinding with a homogenizer in 1/10 M phosphate

Table 1. Distribution of $^{14}CH_2$-carboxymethylphenomycin after the subcutaneous injection of 50 mg/kg to mice

Organs	1 hour (mcg/g)	6 hours (mcg/g)	Organs	1 hour (mcg/g)	6 hours (mcg/g)
Liver	6.2	6.4	Stomach	8.9	3.9
Kidney	1516.9	1411.6	Small intestine	9.9	7.0
Spleen	8.8	5.0	Large intestine	7.4	5.3
Uterus	12.8	4.8	Skin	20.9	7.3
Urinary bladder	24.0	12.3	Muscle	12.3	3.6
Lung	16.3	5.2		(mcg/total)	(mcg/total)
Heart	10.7	2.3	Feces	22.3	64.2
Eye	22.3	2.7	Blood	1.7	2.2
Tongue	11.8	3.2	Urine	112	630
Diaphragm	13.9	5.2			
Peritoneum	30.8	4.3			

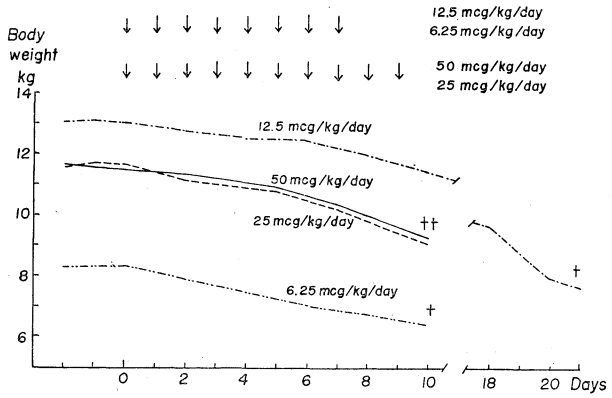
Table 2. Subacute toxicity of phenomycin to 5 mice (i. v. for 14 days)

Dose mcg/kg/day	Number of dead mice at various periods (days)																Total death
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
1,600				2	2	1											5/5
400										1		1		1			3/5
100																	0/5
25																	0/5

buffer (pH 6.8). The amount of $^{14}CH_2$-MCMP in the supernatants was determined by liquid scintillation counting and the amount of $^{14}CH_2$-MCMP in the precipitates was determined after hydrolysis in sealed tubes at 100°C for 17 hours with constant boiling hydrochloric acid. The concentration of MCMP (mcg/g) in various tissues and organs is shown in Table 1. The kidney contained 1.5 mg/g of MCMP (38 % of the injected MCMP) and 6 % of the total MCMP was excreted in urine after 1 hour. Thirty-five per cent of the total MCMP was detected in the kidney (1.4 mg/g) and 32 % of the total MCMP was excreted in urine after 6 hours. More than 20 mcg/g of MCMP was detected in urinary bladder, peritoneum and skin at 1 hour after the injection.

Phenomycin at 1,600 mcg/kg/day, 400 mcg/kg/day, 100 mcg/kg/day and 25 mcg/kg/day was intravenously injected into each 5 mice for 14 days to study the subacute toxicity of phenomycin. As shown in Table 2, all 5 mice survived daily injections of 100 mcg/kg/day. Nevertheless, male dogs could not tolerate intravenous injections of 12.5 mcg/kg/day and 6.25 mcg/kg/day for 8 days as shown in Fig. 1.

Fig. 1. Subacute toxicity of phenomycin in male dog.



Experimental

Carboxymethylation of Phenomycin

An aqueous solution (3 ml) of phenomycin (100 mg=0.01 mM) and monoiodoacetic acid (8 mg=0.043 mM) was adjusted to pH 7.0 by addition of 0.1 N NaOH and kept at 25°C.

After 68 hours, pH of the solution was 6.2 and additional monoiodoacetic acid (5 mg= 0.027 mM) was added. The solution was again adjusted to pH 7.0 and kept for 24 hours at 25°C. Thenafter, the reaction mixture was applied to a column of CM-cellulose (37 cm×1.5 cm diameter) and the column was eluted with $HCOONH_4$ solution with a gradient concentration from 0 to 0.6 M (total 460 ml). The eluate was collected in 9.0-ml fractions and the fractions showing positive PONCEU 3R reaction on a cellulose acetate film were separately dialyzed against distilled water in cellophane tubes for 2 hours and lyophilized. The residues were subjected to electrophoresis on a cellulose acetate film using 0.1 N Tris-HCl buffer (pH 9.0) at 8 mA for 15 minutes to examine their purity and the reaction products were detected by PONCEU 3R reaction. A mixture of *di-*, *tri-* and a trace of *tetra*-carboxymethylphenomycin (21 mg) was recovered from fractions 19~21, monocarboxymethylphenomycin with some dicarboxymethylphenomycin (32 mg) from fractions 22~25, monocarboxymethylphenomycin contaminated with a trace of dicarboxymethylphenomycin (24 mg) from fractions 26~31, a mixture of monocarboxymethylphenomycin and phenomycin (12 mg) from fractions 32~36 and unchanged phenomycin (4 mg) from fractions 34~39. Monocarboxylmethylphenomycin contaminated with a trace of dicarboxymethylphenomycin (23 mg) above obtained was chromatographed on a CM-cellulose column (27 cm ×1.5 cm diameter) eluted with $HCOONH_4$ solution of a gradient increasing concentration using H_2O (230 ml) and 0.3 M $HCOONH_4$ (230 ml, pH 8.8). The eluate was collected in 7.5-ml fractions. Fractions 30~33 gave a mixture of mono- and di-carboxymethylphenomycin, and pure monocarboxymethylphenomycin (12 mg) was recovered from fractions 34~37 by dialysis against water and lyophilization.

Gradient column chromatography on CM-cellulose (27 cm×1.5 cm, diameter) using H_2O (230 ml) and 0.2 M $HCOONH_4$ (230 ml, pH 8.8) was applied to the mixture of *di-*, *tri-* and a trace of *tetra*-carboxymethylphenomycin (20 mg) and the eluate was collected in 7.5-ml fractions. A mixture (12 mg) of *di-*, *tri-* and a trace of *tetra*-carboxymethylphenomycin was recovered from fractions 21~24 and pure dicarboxymethylphenomycin (5 mg) showing one spot by electrophoresis was recovered from fractions 25~27 by the same procedures as above described.

$^{14}CH_2$-Carboxymethylation of Phenomycin

An aqueous solution (2 ml, pH 8.5) of phenomycin (50 mg=0.005 mM) and $^{14}CH_2$-monoiodoacetic acid (2.6 mg=0.014 mM, 15.5 mCi/mM) was kept at 30°C for 92 hours. The radioactive carboxymethylphenomycin mixture was separated into each component by the same procedure as described. Monocarboxymethylphenomycin (15.8 mg), dicarboxymethylphenomycin (2.6 mg) and a mixture of *di-* and *tri*-carboxymethylphenomycin (5.7 mg) were recovered from the above reaction mixture. The radioactivity of $^{14}CH_2$-monocarboxymethylphenomycin was shown to be 1,647 cpm/mcg by the Aloka windowless gas flow counter (Nippon Musen Co.) and 2,450 dpm/mcg by the Beckman liquid scintillation counter LS-250. The activity of $^{14}CH_2$-dicarboxymethylphenomycin shown by the gas flow counter was 3,173 cpm/mcg.

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